Lysophosphatidic Acid Decreases Epidermal Growth Factor Receptor Binding in Airway Epithelial Cells

Karen M. Kassel, Nancy A. Schulte, Stacey M. Parker, Aaron D. Lanik, and Myron L. Toews

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska

Received January 25, 2007; accepted July 18, 2007

ABSTRACT

We showed previously that treatment of human airway smooth muscle cells and lung fibroblasts with lysophosphatidic acid (LPA) increases the binding of epidermal growth factor (EGF) to EGF receptors (EGFRs). The purpose of this study was to determine whether LPA also regulates EGFR binding in airway epithelial cells. Airway epithelial cells were incubated in the absence or presence of 10 μM LPA for increasing times, and binding of 125I-EGF to intact cells on ice was measured. Exposure to LPA for only 15 min caused a 30 to 70% decrease in EGFR binding in a dose-dependent manner, depending on the cell line. This decrease in binding was sustained to at least 18 h in BEAS-2B and primary human bronchial epithelial cells. In contrast, the LPA-induced decrease in binding reversed rapidly in two lung cancer epithelial cell lines, H292 and A549, returning to control levels within 3 h. LPA increased phosphorylation of the EGFR in BEAS-2B cells, and this phosphorylation was inhibited by both 4-(3-chloroanilino)-6,7-dimethoxy-quinazoline (AG1478; EGFR tyrosine kinase inhibitor) and N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001; matrix metalloproteinase inhibitor) but not by CRM197 (heparin-binding EGF inhibitor). AG1478 and GM6001 also inhibited the LPA-induced decrease in EGFR binding but only by 50%, suggesting only partial involvement of EGFR transactivation in the decrease in EGFR binding. In summary, LPA stimulates a decrease in EGFR binding in airway epithelial cells that is sustained in normal cells but that rapidly reverses in cancer cells. LPA-induced transactivation of EGFRs occurs and contributes to the decrease in EGFR binding, but additional pathway(s) may also be involved.

Asthma is a chronic inflammatory disease that results from a combination of increased susceptibility to bronchial epithelial injury and excess tissue repair leading to structural remodeling of the airway wall, perhaps due to an imbalance in responses to growth factors such as epidermal growth factor (EGF) (Holgate et al., 2003; Munakata, 2006). This remodeling includes thickening of the lamina reticularis, mucus gland and smooth muscle cell hypertrophy and hyperplasia, and increased angiogenesis (Lazaar and Panettieri, 2003). These airway wall changes also contribute to the airway hyper-responsiveness that is the hallmark of asthma (Munakata, 2006).

The EGFR receptor (EGFR) has been shown to be involved in many of the processes of airway remodeling and asthma, including mucin synthesis and secretion, goblet cell metaplasia, and sustaining a repair phenotype in the injured epithelium (Puddicombe et al., 2000; Nadel, 2001; Holgate et al., 2003). Asthmatic airways exhibit increased EGFRs in bronchial epithelium and smooth muscle as well as in the basement membrane and glandular cells, compared with normal controls (Amishima et al., 1998). Furthermore, the thickness of the lamina reticularis is positively correlated with EGFR phosphorylation in responses to growth factors such as epidermal growth factor (Holgate et al., 2003; Munakata, 2006).
expression (Puddicombe et al., 2000). EGF has also been shown to stimulate proliferation of human airway smooth muscle (HASM) cells and lung fibroblasts (Cerutis et al., 1997; Ediger and Toews, 2000).

The simple lipid mediator lysophosphatidic acid (LPA) stimulates proliferation, differentiation, tumor metastasis, and cytoskeletal rearrangement in multiple cell types (Wang et al., 2003; Zhao et al., 2006). LPA is released from activated platelets at sites of injury (Eichholtz et al., 1993) where it then contributes to wound repair (Lee et al., 2000), but it can also be synthesized by epithelial cells via the conversion of phosphatidic acid by phospholipase A2/A2 (Cummings et al., 2002). LPA is increased in allergen-challenged lungs compared with saline controls (Georas et al., 2007), and it is 2-to 5-fold higher in injured, prefibrotic mouse lungs (Toews et al., 2004). LPA also increases interleukin-8 production in human bronchial epithelial cells (hBECs) (Cummings et al., 2004), which then acts as a neutrophil chemotacticant to enhance inflammation (Saatian et al., 2006). LPA has been shown to enhance the contractility (Toews et al., 1997) and to stimulate the proliferation of both airway smooth muscle cells (Cerutis et al., 1997) and lung fibroblasts (Ediger and Toews, 2000) as well as to stimulate fibronectin secretion (Romberger et al., 1993) and filopodia extension (Beckmann et al., 1995) by airway epithelial cells. Together, these effects led us to propose an important role for LPA in airway remodeling and disease (Toews et al., 2002). Consistent with this hypothesis, three recent studies have reported significant protection of LPA receptor knockout mice from multiple components of airway disease (Shea et al., 2007; Tager et al., 2007; Zhao et al., 2007).

Studies from our laboratory have shown that LPA stimulates HASM cell proliferation on its own, synergizes with EGF to greatly enhance HASM cell proliferation (Cerutis et al., 1997; Ediger and Toews, 2000), and increases EGFR binding and protein expression (Ediger et al., 2002). LPA does not stimulate transactivation of the EGFR in HASM cells (Ediger et al., 2002), but it does stimulate transactivation of the EGFR in primary human bronchial epithelial cells (Zhao et al., 2006). Two pathways through which LPA can transactivate the EGFR have been established. “Ligand-dependent” transactivation involves the activation of matrix metalloproteinases (MMPs) (Shida et al., 2005; Mori et al., 2006), the cleavage of EGFR proligands (Zhao et al., 2006; Xu et al., 2007), and the subsequent activation of the EGFR. “Ligand-independent” transactivation involves the intracellular activation of EGFRs by kinases such as Src (Daub et al., 1997; Shah et al., 2005).

The purpose of this study was to examine LPA regulation of EGFR binding in airway epithelial cells and to determine whether LPA-mediated transactivation of the EGFR plays a role in this regulation. Because of the contribution of EGF and the EGFR to epithelial repair in asthma, determining how LPA and other factors that are elevated in asthmatic airways regulate the EGFR is critical for understanding the role of the EGFR in airway remodeling. These studies also define additional regulatory interactions between GPCRs and receptor tyrosine kinases in airway epithelial cells. It is noteworthy that these interactions are different for airway epithelial cells than for smooth muscle cells and fibroblasts, and they are also different between the normal and the lung cancer cell lines tested.

Materials and Methods

Reagents. Cell culture medium, fetal bovine serum (FBS), penicillin/streptomycin (Pen/Strep), Tris-glycine gels, and polyvinylidene difluoride-FL membranes were obtained from Invitrogen (Carlsbad, CA). Vitrogen was purchased from Angiotech Biomaterials (Palo Alto, CA). EGF was purchased from BioSource International (Camarillo, CA), and LPA, 1-O-octadecyl-2-hydroxy-sn-glycero-3-phospho-ether (ether LPA), phosphatidic acid, and lysophosphatidylcholine were from Avanti Polar Lipids (Alabaster, AL). GM6001 was from BIOMOL Research Laboratories (Plymouth Meeting, PA), and AG1478 and diphertheria toxin CRM mutant (CRM197) were from Calbiochem (San Diego, CA). EGFR and phosphothypoxarine (pY99) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit 800 antibody was from Rockland (Gilbertsville, PA), and goat anti-mouse 680 antibody was from Invitrogen. Other chemicals were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). 125I (Amersham, Piscataway, NJ) was used to synthesize 125I-EGF by a chloramine T protocol, and the 125I-EGF was purified on a Sephadex G-25 column (Rizzino et al., 1988).

Cell Culture. BEAS-2B, H292, and A549 cells were from American Type Culture Collection (Manassas, VA). Primary cultures of hBECs were kindly provided by Dr. Stephen Rennard (University of Nebraska Medical Center, Omaha, NE). BEAS-2B cells and hBECs were cultured in a 1:1 mixture of L-15 and RPMI-1640 medium (Lechner and Laveck, 1985). BEAS-2B cells and hBECs were cultured in Ham's F-12 medium overnight. H292 cells were cultured in RPMI-1640 medium with 10% FBS and 1% Pen/Strep, and A549 lung cancer epithelial cells were cultured in Ham's F-12 with 10% FBS and 1% Pen/Strep. All cells were maintained in a humidified 5% CO2 incubator at 37°C and passed weekly.

125I-EGF Binding Assays. BEAS-2B cells and H292 cells were plated at 50,000 cells/well in six-well plates and grown to confluence. A549 cells were plated at 100,000 cells/well in six-well plates and grown to confluence. Cells were starved in the appropriate basal medium (without FBS or other growth factors) overnight (18–24 h), and then they were treated with LPA or other agents for the indicated times, usually 15 min or 18 h. Control wells received bovine serum albumin (BSA), because LPA was dissolved in 0.25% BSA. If cells were treated with an inhibitor, the inhibitor was added 30 min before stimulation with LPA. Cells were then washed once with 2 ml of 37°C DMEM-HEPES with 0.1% BSA. Cells were then washed once with 2 ml of ice-cold DMEM-HEPES with 0.1% BSA, and then incubated with 125I-EGF for 4 h on ice to selectively label cell surface EGFRs and to prevent EGFR endocytosis and EGF degradation. The 125I-EGF solution contained approximately 300,000 cpm/ml in DMEM-HEPES with 0.1% BSA. Nonspecific binding was defined by the addition of 300 ng/ml nonradioactive EGF. After 4 h of binding on ice, the cells were washed four times with 2 ml of ice-cold DMEM-HEPES with 0.1% BSA, and then they were dissolved in 1 ml of 0.2 N NaOH. The 125I was then quantified in a gamma counter.

EGFR Transactivation Assays. BEAS-2B cells were plated on 60-mm dishes, and cells were grown to confluence. Cells were then starved in RPMI 1640 medium + Pen/Strep overnight. For experiments with inhibitors, cells were pretreated with the inhibitor for 30 min before stimulation with vehicle or 10 μM LPA for 2 min in the continued presence of inhibitor. Cells were then washed with PBS, incubated on ice for 10 min in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, and 10 μl/mL protease inhibitor cocktail), and lysed by scraping. The cell lysate was centrifuged for 5 min at 15,300 rpm at 4°C, and the supernatant was diluted in sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue) and boiled for 5 min. Samples were loaded onto 8% or 10% gel, and electrophoresed for 110 min at 120 V. Gels for EGFR blots were transferred with a semi-wet apparatus to polyvinylidene difluoride-FL mem-
brane for 2 h at 26 V. Membranes were then blocked for 1 h with a 1:1 mixture of PBS/Odyssey blocking buffer (Li-Cor, Lincoln, NE). EGFR blots were incubated with pY99 (rabbit) and EGFR (mouse) antibodies overnight at 4°C. Blots were washed five times with Tris-buffered saline + 0.1% Tween 20 for 5 min each, and then they were incubated with secondary anti-rabbit (680 nm) and anti-mouse (800 nm) antibodies for 1 h before washing again with Tris-buffered saline + 0.1% Tween 20 as described above. Blots were then scanned and quantified using the Odyssey infrared imaging system (Li-Cor). Data are expressed as the ratio of phosphorylated/total protein, and then further normalized as the ratio of treated/control cells.

Data Analyses. All data are presented as means ± S.E.M. Data were analyzed using GraphPad Prism 4.00 (GraphPad Software, Inc., San Diego, CA). Unless otherwise indicated, all data shown are representative of at least three separate experiments. t tests were used to determine statistical significance.

**Results**

**LPA Induces a Rapid and Sustained Decrease in EGFR Binding in Normal Epithelial Cells.** BEAS-2B cells, which are virally transformed and immortalized but frequently used as a model for normal airway epithelial cells, and hBECs from primary cultures were treated with 10 μM LPA or with BSA-containing vehicle for various times, and then they were incubated with 125I-EGF on ice to quantify cell surface EGF receptor binding. In contrast to the up-regulation of EGFRs that occurs in airway smooth muscle cells (Ediger et al., 2002), LPA induced a rapid decrease in EGFR binding in these epithelial cells, with the maximal decrease occurring by 15 min. After 15 min of treatment, BEAS-2B cells showed a decrease of 32 ± 2%, and hBECs showed a decrease of 26 ± 14% (Fig. 1A). This decrease in binding was sustained through 18 h of continuous LPA exposure, with decreases of 45 ± 5 and 44 ± 9% for BEAS-2B cells and hBECs, respectively.

**LPA Induces a Rapid but Transient Decrease in EGFR Binding in Lung Cancer Epithelial Cells.** H292 and A549 lung cancer epithelial cells were treated with 10 μM LPA for various times, and then they were incubated with 125I-EGF on ice as in the studies with normal cells described above. These cancer cell lines showed a greater extent of rapid decrease at 15 min; LPA decreased EGFR binding by 54 ± 4% in H292 cells and by 67 ± 2% in A549 cells after 15 min of treatment. However, in contrast to the normal epithelial cells, this decrease was not sustained (Fig. 1B). After only 1 h of treatment with LPA, EGFR binding began to return to control levels, and by 18 h, EGFR binding had returned to baseline in H292 cells (90 ± 3% control), and it was increased above baseline in A549 cells (113 ± 5% control) (Fig. 1B). The reversal of the initial decrease in binding occurred somewhat more rapidly for H292 cells than for A549 cells.

**Concentration Dependence of the LPA-Induced Changes in EGFR Binding.** The LPA concentration dependence of the changes in EGFR binding was determined at both 15 min and 18 h for BEAS-2B, H292, and A549 cells (Fig. 2, A–C). In BEAS-2B cells, the LPA-induced decrease in EGFR binding at 15 min occurred with high potency; however, the dose-response curve was shifted to the right after 18 h of treatment. The EC50 values were 3.8 ± 0.2 nM at 15 min (Fig. 2A) and 1.1 ± 0.1 μM at 18 h (Fig. 2B). To test whether the lower potency at 18 h was due to deacylation of LPA, we tested the effects of ether LPA, which has a stable ether bond between C1 of the glycerol backbone and the fatty acid chain, preventing phospholipase-mediated deacylation (Prestwich et al., 2005). The dose-response curves for LPA and ether LPA were very similar at both 15 min and 18 h, with EC50 values of 9.8 ± 0.8 nM at 15 min and 1.9 ± 0.1 μM at 18 h for ether LPA (Fig. 2C). Thus, phospholipase degradation of the LPA is apparently not the explanation for the lower potency of LPA for the 18-h decrease.

**The Decrease in Binding Results Primarily from a Decrease in Bmax.** To assess the nature of the decrease in EGFR binding, saturation and competition binding assays were performed after exposure to 10 μM LPA for 15 min for the two prototype cell lines, BEAS-2B for normal cells and H292 for cancer cells. Saturation curves with both cell lines revealed a prominent decrease in Bmax after LPA treatment; a small increase in Kd was also observed, but it was not sufficient to make a major contribution to the decrease in saturation. A similar decrease in Bmax with a small increase in Kd was also observed for A549 cells after LPA treatment.
Effects of Other GPCR Agents on EGFR Binding. The effects on EGFR binding were assessed for two additional GPCR agents, sphingosine-1-phosphate (S1P) and thrombin, at both 15 min and 18 h. Both of these agonists are implicated in airway disease, and they can activate similar GPCR pathways as LPA (Jolly et al., 2002; Rosenfeldt et al., 2003; Terada et al., 2004; Kume et al., 2007). With 15-min treatments, the lysophospholipid mediator S1P was similarly potent, but it was slightly more efficacious than LPA in both BEAS-2B and A549 cells, with decreases of 52 ± 4 and 69 ± 3% and EC50 values of 3.6 ± 0.6 and 35 ± 4 nM, respectively (Fig. 4, A and C). S1P was less potent and equally efficacious as LPA in H292 cells, with a decrease of 38 ± 2% and an EC50 value of 2.0 ± 0.1 μM (Fig. 4B).

Thrombin was also able to decrease EGFR binding after 15 min of treatment, similar to the effects of LPA and S1P. As expected, the peptide thrombin was much more potent than LPA or S1P, with EC50 values of 6.3 ± 0.7 pM and 1.1 ± 0.1 nM in BEAS-2B and A549 cells, respectively (Fig. 4, A and C); maximal effects of thrombin were similar to those with LPA and S1P. Thrombin seemed to decrease EGFR binding in H292 cells, but the potency was too low to establish a full dose-response curve and EC50 values (Fig. 4B).

S1P and thrombin also induced similar changes as LPA after 18 h of treatment. In BEAS-2B cells, S1P was more potent but slightly less efficacious than LPA, with a decrease of 30 ± 4% and an EC50 value of 200 ± 40 nM (Fig. 5A). In A549 cells, both S1P and thrombin mimicked the small increase in EGFR binding seen after prolonged LPA treatment, with increases of 44 ± 5 and 35 ± 18%, respectively. Thrombin was the most potent agent for this effect, with an EC50 of 5.8 ± 0.4 nM, followed by S1P and LPA, with EC50 values of 1.5 ± 0.3 and 1.4 ± 0.4 μM, respectively (Figs. 2B and 5C). Similar to LPA, S1P and thrombin had no effect on EGFR binding after 18 h of treatment in H292 cells (Fig. 5B).

Effects of LPA-Related Agents on EGFR Binding. The specificity for LPA of these effects on EGFR binding was assessed by testing several structurally related compounds. Oleic acid is an 18:1 fatty acid identical to the single fatty acid chain on the 18:1 (oleoyl) LPA used in these studies; oleic acid had no effect on EGFR binding in any of the cell types tested at either 15 min or 18 h (Table 1). Lyso-phosphatidylcholine (LPC) contains a choline head group attached to the phosphate rather than the free phosphate of LPA. LPC had no effect on any cell type after 18 h of treatment, but it showed weak effects in all cell types after 15 min of treatment (Table 1). Phosphatidic acid (PA) contains fatty acid chains on both C1 and C2 of the glycerol backbone instead of the single fatty acid on C1 for LPA. In contrast to oleic acid and LPC, PA did decrease EGFR binding in both BEAS-2B and A549 cells at 15 min, although with markedly lower potency than LPA. PA also exhibited a sustained decrease in EGFR binding in BEAS-2B cells with approximately the same potency as LPA. PA had no effect in H292 cells at 15 min or 18 h, and it also had no effect on A549 cells at 18 h (Table 1).

Effects of Different Fatty Acid Chain Lengths. Oleoyl-LPA (18:1 LPA) is the most commonly studied form of LPA. We tested both stearoyl (18:0) LPA and palmitoyl (16:0) LPA to determine the LPA structure-activity relationship for the decrease in EGFR binding. The 18:1 LPA was the most potent and most efficacious agent after 15 min of treatment, followed by 18:0 LPA (Table 1). 16:0 LPA was the least potent at 15 min, but it was as effective at decreasing EGFR binding as 18:0 LPA in all cell types. Interestingly, 18:0 LPA was the most potent for the sustained decrease in binding after 18 h of treatment in BEAS-2B cells, followed by 18:1 LPA and then 16:0 LPA (Table 1). This potency order was reversed in A549 cells, where EGFR binding increases rather than decreases after 18 h of treatment, with 16:0 LPA being the most potent, followed by 18:1 LPA, and with 18:0 LPA being the least potent.
LPA Stimulates Tyrosine Phosphorylation of the EGFR in BEAS-2B Cells. LPA transactivates the EGFR in many cells, including primary human bronchial epithelial cells (Zhao et al., 2006). The ability of LPA to transactivate EGFRs in BEAS-2B cells was assessed by measuring EGFR tyrosine phosphorylation (Fig. 6). BEAS-2B cells were treated for the indicated times in the presence of 10 \( \mu \)M LPA. LPA stimulated EGFR phosphorylation 1.7- to 2.0-fold, and this phosphorylation was sustained to at least 15 min. Although LPA stimulated EGFR phosphorylation much less than EGF itself, this magnitude is similar to what has been reported previously (Zhao et al., 2006).

AG1478 and GM6001 Inhibit EGFR Phosphorylation in BEAS-2B Cells. To assess the possible role of transactivation in the decrease in EGFR binding, inhibitors were used to block the LPA-stimulated transactivation. The EGFR tyrosine kinase inhibitor AG1478, the MMP inhibitor GM6001, and CRM197, a nontoxic mutant of diphtheria toxin that specifically binds heparin-binding (HB)-EGF, were used to test the mechanism of transactivation. BEAS-2B cells were pretreated with 2.5 \( \mu \)M AG1478, 25 \( \mu \)M GM6001, or 10 \( \mu \)g/ml CRM197 before cell treatment for 2 min with BSA or LPA. Western blotting for total and phospho-EGFR showed that both AG1478 and GM6001 completely inhibited phosphorylation of the EGFR by LPA (Fig. 7); thus, MMP activation and the kinase activity of the EGFR itself are required for transactivation by LPA. However, CRM197 did not have any effect on the transactivation (Fig. 7), suggesting that an EGFR proligand other than HB-EGF is cleaved to stimulate transactivation.

AG1478 and GM6001 Inhibit the Rapid Decrease in Binding by LPA in BEAS-2B Cells. To determine whether transactivation contributes to the LPA-induced decrease in EGFR binding, BEAS-2B cells were pretreated for 30 min with 2.5 \( \mu \)M AG1478 or 25 \( \mu \)M GM6001 before treatment for 15 min or 18 h with 10 \( \mu \)M LPA in the continued presence of the inhibitor. Cells were then washed and incubated for 4 h on ice with \( ^{125} \)I-EGF to label cell surface EGFRs. AG1478 caused an increase in EGFR binding of approximately 2-fold, similar to data reported previously with human airway smooth muscle cells (Ediger et al., 2002). In the absence of AG1478 or GM6001, LPA significantly decreased EGFR binding by approximately 30% after 15 min. In the presence of AG1478 or GM6001, LPA only decreased binding by 14 \( \pm \) 8 and 12 \( \pm \) 3\% (\( \beta \)), respectively (Fig. 8, A and B), suggesting at least partial involvement of EGFR transactivation in the LPA-induced rapid decrease in EGFR binding. AG1478 and GM6001 had minimal effects on the sustained decrease in binding, suggesting that an additional mechanism is involved in the sustained decrease. The HB-EGF inhibitor CRM197 did not inhibit the LPA-induced decrease in binding at 15 min or 18 h (Fig. 8C), consistent with its inability to inhibit transactivation in BEAS-2B cells (Fig. 7). To determine whether Src-mediated ligand-independent transactivation might be responsible for the portion of the decrease in binding that is not mediated by ligand-dependent transactivation, BEAS-2B cells were treated with the Src inhibitors PP1 and PP2. Neither PP1 nor PP2 had any effect on the rapid or sustained decreases in binding (data not shown), indicating that Src-mediated ligand-independent transactivation is not involved in the LPA-induced decrease in EGFR binding in BEAS-2B cells.

Discussion

The results presented here show that LPA regulates EGFR binding in human airway epithelial cells in ways that are both complex and markedly different from effects in airway mesenchymal cells. LPA caused a rapid decrease in cell surface EGFR binding in all of the airway epithelial cells tested, with maximal decreases occurring within 15 min. This decrease in binding was sustained to at least 18 h in the two cell types used as models of normal airway epithelium, BEAS-2B cells and hBECs, but it reversed rapidly in the two lung cancer cell lines tested, H292 and A549 cells. Dose-response curves showed that the LPA-induced decrease in EGFR bind-
ing after 15 min of treatment occurs to a greater extent but with lower potency in the lung cancer epithelial cells compared with the normal epithelial cells. Whether these potency differences relate to different LPA receptor subtypes, differential metabolism of LPA, or differential regulation of EGFRs among these cells remains to be established.

Compounds with structural similarities to LPA, such as oleic acid and LPC, either had no effect on EGFR binding or were much less potent than LPA. PA also had either no effect or was much less potent than LPA, with the exception of 18-h treatment of BEAS-2B cells, where PA and LPA had similar potency and efficacy. The difference in the dose-response curves to LPA at 15 min and 18 h in BEAS-2B cells is not due to deacylation of LPA by phospholipases, but the decreased potency of LPA after 18 h of treatment could result from the metabolism of LPA by other enzymes, thereby decreasing the amount of LPA available to regulate EGFR binding. The specificity of these effects suggests that the decreases in EGFR binding for LPA versus structural analogs is mediated by LPA signaling through one or more of its specific GPCRs and not via a nonspecific lipid-mediated effect.

The decrease in EGFR binding is a result of a decrease in EGF binding sites after 15 min of LPA treatment, as indicated by both saturation and competition curves; there is also a small decrease in the affinity of EGF for its receptor, although this change in affinity contributes minimally to the decrease in EGFR binding. Because the binding studies were conducted with cells on ice to selectively label cell surface receptors, the decrease in binding could be due to receptor internalization or some other decrease in receptor accessibility, without an actual loss of EGFR protein. One obvious
TABLE 1

Other LPA-related agents decrease EGFR binding in airway epithelial cells

<table>
<thead>
<tr>
<th></th>
<th>Change in Binding</th>
<th>EC50</th>
<th>Change in Binding</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15 min</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEAS-2B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 LPA</td>
<td>-38 ± 4**</td>
<td>4 ± 0.8</td>
<td>-47 ± 10*</td>
<td>1100 ± 240</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>5 ± 4</td>
<td>N.A.</td>
<td>10 ± 5</td>
<td>N.A.</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>-34 ± 3**</td>
<td>120 ± 24</td>
<td>-72 ± 4***</td>
<td>900 ± 100</td>
</tr>
<tr>
<td>Lysophosphatidyl choline</td>
<td>-20 ± 5*</td>
<td>2600 ± 1800</td>
<td>-10 ± 5</td>
<td>N.A.</td>
</tr>
<tr>
<td>18:0 LPA</td>
<td>-33 ± 1**</td>
<td>27 ± 8</td>
<td>-56 ± 7**</td>
<td>580 ± 130</td>
</tr>
<tr>
<td>16:0 LPA</td>
<td>-33 ± 5*</td>
<td>32 ± 8</td>
<td>-55 ± 8**</td>
<td>1900 ± 500</td>
</tr>
<tr>
<td>H292</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 LPA</td>
<td>-42 ± 3***</td>
<td>170 ± 30</td>
<td>-8 ± 2**</td>
<td>1700 ± 1200</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>4 ± 3</td>
<td>N.A.</td>
<td>0.1 ± 2</td>
<td>N.A.</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>-6 ± 7</td>
<td>N.A.</td>
<td>-9 ± 3*</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lysophosphatidyl choline</td>
<td>-19 ± 4*</td>
<td>&gt;10,000</td>
<td>1.0 ± 3</td>
<td>N.A.</td>
</tr>
<tr>
<td>18:0 LPA</td>
<td>-31 ± 3**</td>
<td>250 ± 50</td>
<td>-7 ± 8</td>
<td>N.A.</td>
</tr>
<tr>
<td>16:0 LPA</td>
<td>-31 ± 5**</td>
<td>1600 ± 600</td>
<td>-6 ± 6</td>
<td>N.A.</td>
</tr>
<tr>
<td>A549</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 LPA</td>
<td>-60 ± 3**</td>
<td>24 ± 4</td>
<td>31 ± 8*</td>
<td>1500 ± 940</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>-4 ± 3</td>
<td>N.A.</td>
<td>1 ± 4</td>
<td>N.A.</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>-27 ± 2**</td>
<td>530 ± 120</td>
<td>8 ± 3</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lysophosphatidyl choline</td>
<td>-20 ± 3**</td>
<td>810 ± 370</td>
<td>10 ± 2*</td>
<td>N.A.</td>
</tr>
<tr>
<td>18:0 LPA</td>
<td>-34 ± 2**</td>
<td>530 ± 140</td>
<td>26 ± 15</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>16:0 LPA</td>
<td>-27 ± 3**</td>
<td>1200 ± 400</td>
<td>15 ± 5*</td>
<td>860 ± 370</td>
</tr>
</tbody>
</table>

* P ≤ 0.05 compared with control.
** P ≤ 0.01 compared with control.
*** P ≤ 0.001 compared with control.

Fig. 6. LPA transactivates the EGFR in BEAS-2B cells. BEAS-2B cells were treated with 10 μM LPA for the indicated times. Cells were then lysed, and cell lysates were separated by SDS-polyacrylamide gel electrophoresis and blotted for total EGFR (green) and pY99 (red). Western blots were scanned and quantified using the Odyssey infrared imaging system. Data are shown as phospho-EGFR/total EGFR and then as a fold of time 0. Top, representative blot. Bottom, data from three to four separate experiments.

Fig. 7. LPA stimulates transactivation via MMPs in BEAS-2B cells. BEAS-2B cells were pretreated for 30 min with 2.5 μM AG1478, 25 μM GM6001, or 10 μg/ml CRM197, and then they were treated with vehicle or 10 μM LPA for 2 min. Cells were then lysed, and cell lysate was separated by SDS-polyacrylamide gel electrophoresis and blotted for total EGFR and phosphotyrosine. Western blots were scanned and quantified using the Odyssey Infrared Imaging System. Data are shown as phospho-EGFR/total EGFR and then as a fold of vehicle-treated values. Data are from at least four separate experiments, except for CRM197 (n = 2).

The possibility is that the rapid decrease in binding is a result of transactivation and subsequent internalization of EGFRs induced by LPA. Previous studies have established that LPA transactivates the EGFR in primary human bronchial epithelial cells (Zhao et al., 2006) and that transactivation of the EGFR results in receptor internalization and a decrease in 125I-EGF binding on ice in some cell types (Pierce et al., 2000; Kim et al., 2003; Olivas-Reyes et al., 2005). In rat hepatic C9 cells, angiotensin II stimulates a decrease in EGFR cell surface binding via transactivation and internalization of the EGFR (Olivares-Reyes et al., 2005). Similar results were obtained with serotonin in rat renal mesangial cells (Grewal...
In summary, LPA rapidly decreases EGFR binding in airway epithelial cells, with a sustained decrease in two models of normal epithelial cells but only a transient decrease in two lung cancer cell lines. The rapid decrease in binding is par-
LPA Regulation of Airway Epithelial Cell EGFR Receptors

References


SIMPLIFIED: LPA binding is a key process in airway remodeling, and understanding it can help in developing new treatments.


**Address correspondence to:** Dr. Myron L. Toews, 985800 Nebraska Medical Center, Omaha, NE 68198-5800. E-mail: mtoews@unmc.edu